Acidic Free Amino Compounds Formed in Various Lactic Acid Starter Cultures as Measured by Ion Exchange Chromatography[1, 2]

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The role of starter organisms in the ripening of various cheese varieties has been the subject of extensive study in recent years (Deane, 1951; Peterson *et al.*, 1948; Baribo and Foster, 1952; and Van der Zant and Nelson, 1954a). The accumulation of various free amino compounds during cheese ripening has been attributed primarily to enzymes of bacterial origin (Peterson *et al.*, 1948; Deane, 1951; Harper and Gould, 1952).

Most of the previous studies have been limited to the organism *Streptococcus lactis* (Hansen, 1941; Morgan and Nelson, 1951). Baribo and Foster (1952) determined some of the characteristics of the endocellular proteinases of one strain each of *S. lactis*, *Lactobacillus casei*, and *Micrococcus freudenreichii*. Van der Zant and Nelson (1953a and 1953b) investigated the proteolytic activity of *S. lactis* and a cell-free extract of this organism prepared by sonic vibration. Recently, Van der Zant and Nelson (1954a, 1954b) reported using paper chromatography to investigate the presence of amino acids and peptides in skimmilk inoculated with *S. lactis*, and to study some of the characteristics of the endocellular peptidases of this organism.

In the present study, ion exchange chromatography was applied to investigate the proteolytic activity as measured by the presence of various acidic free amino acids of several lactic acid starter organisms; namely, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, and *Streptococcus thermophilus*.

Experimental Methods

Cultures. *Lactobacillus bulgaricus* strains V_4, V_{10}, V_{12}, V_{29}, V_{71}, R and R_3, *L. lactis* strains 8 to 15, K_m, V_{104} and V_{109}, *S. thermophilus* strains S, T_3, T_4, and T_5 were used in the present study. These cultures were obtained from the Dairy Products Section, Washington Utilization Research Branch, U. S. Department of Agriculture. They represent isolates from starters used in the manufacture of Provolone and Romano cheeses. They were cultivated through serial passage until they grew actively at 42 C within 12 hours and then were used to inoculate 700 ml of sterile skimmilk using two per cent inoculum. Unless otherwise mentioned, the cultures were incubated for 12 hours at 42 C and then stored at 10 C for various lengths of time.

Application of ion exchange chromatography. The proteolytic activity of these organisms on the skimmilk was measured by determining qualitatively and quantitatively the presence of eleven free acidic α-amino compounds using Moore and Stein's (1951) ion exchange chromatographic method as modified by Hamdy *et al.* (1955). The acids studied in the order of their elution were: cysteic acid, serine phosphate, taurine, aspartic acid, threonine, serine, asparagine, glutamine, glutamic acid, proline, and glycine.

Preparation of sample for ion exchange chromatography. Fifty-ml samples were withdrawn aseptically from the milk cultures with a sterile pipette and placed in 250-ml centrifuge bottles with 100 ml of distilled water containing 20 per cent ethyl alcohol by volume. The mixture was stirred vigorously with a mechanical stirrer for five minutes, and then cooled at 8 C for approximately two hours. This permitted precipitation of the nonsoluble protein fractions, after which the sample was centrifuged and then filtered through a fine sintered glass filter. The precipitates were washed twice with 50 ml distilled water. The filtrate (about 200 ml) was condensed at a low temperature under vacuum to 8 to 10 ml. One ml of this concentrate was adjusted to pH 3.0 and used for the ion exchange chromatography.

The chromatographic separation. The sample was adsorbed on a 110 x 0.9-cm sulfonated polystyrene resin column operated in the sodium form. The column was then mounted on an automatic constant volume (1.0 ml) fraction collector. Two aliquot portions (0.5 ml) of pH 4.0 sodium citrate buffer were used to wash the acids in the resin bed. The chromatogram was developed by displacing these adsorbed amino acids using about 150 ml of pH 4.0 sodium citrate buffer. The effluents were collected in 1.0-ml fractions in small 10-ml glass vials, and the elution was concluded after collecting 150 fractions.

Analysis of effluent fractions. The presence and the

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position of the α-amino acids were determined by spotting, with a capillary tube, a small amount of each of the effluent fractions on Whatman No. 1 filter paper. The spots were completely air dried and sprayed with the ninhydrin-n-propanol solution of Moore and Stein (1948), then placed in a 100 C oven for 3 to 5 minutes to develop the color. After establishing the position of the amino acids and determining their identity by comparison with the location of pure amino acids (Hamdy *et al.*, 1955), the vials containing the specific α-amino compound were combined and then adjusted to a known volume, and the concentration determined using the photometric ninhydrin method of Moore and Stein (1948). The concentration of the specific amino acids in the sample was then calculated using standard curves for each acid as described by Hamdy *et al.* (1955). In all amino acids determinations, duplicate experiments were made and the average results are reported.

Results

This study was divided into two general phases. The first phase consisted of an investigation of the proteolytic activity of single strain cultures of *L. lactis*, *L. bulgaricus* and *S. thermophilus*. For the second phase of work, mixed strain cultures of the three organisms were used.

Single strain studies. *L. lactis* V_{104}, *L. bulgaricus* V_4, and *S. thermophilus* T_5 were used. These strains were grown alone and in various combinations with each other in skimmilk for 12 hours at 42 C and then stored at 10 C. Selection of these specific strains was made primarily on their ability to produce comparable amounts of acid, with a range in pH values of 5.1 to 5.3 after 12 hours incubation. This permitted a study of the effect of one species on another without suppressing the growth of any of the individual organisms.

The results of the analyses of the three different lactic acid producing cultures at the end of 12 hours of incubation and after 0.5, 3, 7, 18, and 28 days of storage are shown in table 1. As noted, neither cysteic acid or proline could be detected in any of the cultures at any given time. Of the other amino compounds, only serine phosphate was present in all cultures at all times. In general, *L. lactis* V_{104} showed the greatest concentration of amino acids at any given time, whereas very few of the amino acids could be found in milk inoculated with *S. thermophilus* T_5. The effect of storage time on the accumulation of the various acids is considerable, and the variations in this effect should

Table 1. *The acidic free amino compound content of various lactic acid starter cultures**

Age of Culture	Concentration of Amino Compounds in mg/1000 ml of Fermented Milk†							
	Serine phosphate	Taurine	Aspartic acid	Threonine ‡	Serine ‡	Asparagine & Glutamine	Glutamic acid	Glycine
Lactobacillus lactis* V *104								
12 hours incubation	41.7	0	+	+	+	0	+	0
12 hours storage	68.3	0	0	7.9		10.1	52.9	0
3 days storage	121.1	2.2	0	0	0	146.0	41.2	0
7 days storage	75.2	17.6	0	0.2		21.4	43.2	39.0
18 days storage	157.9	+	0	139.2		37.7	0	56.6
28 days storage	159.5	0	0	0	8.0	98.0	0	28.1
***Streptococcus thermophilus* T_5**								
12 hours incubation	31.9	0	0	0	0	0	+	+
12 hours storage	45.6	+	0	0	0	0	+	+
3 days storage	52.2	0	0	0	0	0	0	0
7 days storage	49.5	0	0	0	0	0	0	0
18 days storage	49.4	0	0	0	0	0	0	0
28 days storage	45.9	0	0	0	0	0	0	0
***Lactobacillus bulgaricus* V_4**								
12 hours incubation	36.3	0	0.8	0	0	0	+	0
12 hours storage	43.9	3.6	1.9	18.2		0	+	+
3 days storage	97.9	+	0	12.1		0	+	25.2
7 days storage	104.4	0	0	0	9.4	35.8	+	+
18 days storage	97.4	+	0	0	3.8	15.6	0	13.5
28 days storage	146.2	0	0	0	8.0	31.3	0	+

* Acid present but in less than 0.5 mg/1000 ml concentration.

† Cysteic acid and proline were not present.

‡ Threonine and serine could not always be separated, and asparagine and glutamine were not separated in this trial.

TABLE 2. *The acidic free amino compound content of various lactic acid starter cultures*

Age of Culture	Concentration of Amino Compounds in mg/1000 ml of Fermented Milk*							
	Serine phosphate	Taurine	Aspartic acid	Threonine	Serine	Asparagine & Glutamine	Glutamic acid	Glycine
Lactobacillus lactis V104 and *Lactobacillus bulgaricus* V_4								
12 hours storage	100.3	8.7	0	0	0	0	35.3	0
3 days storage	93.9	14.7	0	0	0	199.1	0	0
7 days storage	113.5	0	0	0	0	0	0	29.1
18 days storage	205.1	3.5	0	0	0	38.0	0	51.3
28 days storage	102.3	+†	0	0	0	44.7	0	55.4
Lactobacillus bulgaricus V_4 and *Streptococcus thermophilus* T_5								
12 hours storage	82.0	0	0	0	0	0	0	0
3 days storage	102.4	0	0	0	0	0	0	0
7 days storage	84.3	1.5	0	0	0	0.89	0	67.03
18 days storage	77.3	0	0	0	0	+	0	0
28 days storage	84.3	0	0	0	0	0	0	0
Lactobacillus lactis V104 and *Streptococcus thermophilus* T_5								
12 hours storage	12.5	0	0	0	0	0	5.1	0
3 days storage	31.9	0	0	0	0	57.8	8.8	0
7 days storage	71.0	0	0	0	0	60.3	86.4	0
18 days storage	236.9	+	0	0	0	0	0	53.5
28 days storage	218.7	11.4	22.6	0	0	0	0	97.7
Lactobacillus lactis V104, *Lactobacillus bulgaricus* V_4 and *Streptococcus thermophilus* T_5								
12 hours storage	62.6	+	0	7.4		0	0	28.5
3 days storage	77.3	+	0	14.3		19.5	0	65.6
7 days storage	96.3	+	0	0	5.4	25.8	0	2.6
18 days storage	200.4	3.1	0	0	28.6	74.0	0	74.0
28 days storage	99.0	0	0	0	38.1	56.0	0	34.1

* Cysteic acid and proline were not present.

† Acid present but in less than 0.5 mg/1000 ml concentration.

be noted. For example, aspartic and glutamic acids tended to be highest after 12 hours of storage, then to decrease, whereas the amides, asparagine and glutamine, and glycine increased during the storage period. Other compounds, such as serine phosphate and threonine-serine increased and decreased during the storage.

The various single strains of organisms were also grown in combination with one another and stored in the same manner. As shown in table 2, there is a definite effect of one species on another as indicated by the change in the pattern of free amino compounds produced. For example, glycine, which was produced in very small amounts or not at all, was present in much higher concentrations in the mixed cultures.

Glutamic acid was produced by *L. lactis* V_{104} and by *L. bulgaricus* V_4 up to the 18th and 7th day of storage respectively, yet upon growth and storage of these cultures in combination with one another the glutamic acid was not detected except at 12 hours storage. Serine and threonine also were produced by these strains growing alone, but their presence could not be found when *L. lactis* and *L. bulgaricus* were grown together. The same effect was noted when *L. bulgaricus* V_4 was grown with *S. thermophilus* T_5. Upon the growth of these organisms together, most of the acids produced by *L. bulgaricus* V_4 were not detected.

The combination of all three organisms resulted in an entirely different amino acid pattern than when any two were grown in combination. For example, both threonine and serine were detected after incubation and subsequent storage. Glutamic acid was not found at any time, whereas asparagine and glutamine were present after 3 days of storage. Glycine was present in all samples at all periods of storage, which was not true when any two of the organisms were grown together.

Mixed strain studies. L. bulgaricus strains V_4, V_{10}, V_{12}, V_{29}, V_{71}, R and R_3; *L. lactis* strains K_m, 8–15, V_{104}, and V_{109}; *S. thermophilus* strains S, T_3, T_4, and T_5 were used in this study. These mixed strains were grown alone and in various combinations in skimmilk to investigate their proteolytic activity, indicated by the liberation of amino acids, and the effect of one species on the other as previously noticed in the single strains study. The cultures were grown for 24 hours at 42 C,

TABLE 3. *The acidic free amino acid content of various mixed cultures of lactic acid starter cultures**

Age of Culture	Concentration of Acids in mg/1000 ml of Fermented Milk†					
	Serine phosphate	Taurine	Aspartic acid	Serine	Asparagine	Glutamic acid
Lactobacillus lactis (mixed strains)						
2 days	41.6	0	0	0	0	50.59
12 days	18.6	0	0	0	0	79.12
40 days	59.8	0	0	0	0	26.4
Streptococcus thermophilus (mixed strains)						
2 days	68.9	0	0	0	0	140.7
12 days	97.8	5.92	4.20	0	0	53.9
40 days	172.0	0	0	19.56	0	38.4
Lactobacillus bulgaricus (mixed strains)						
2 days	27.0	0	0	0	0	0
12 days	39.6	0	14.10	0	0	75.3
40 days	76.4	0	0	87.8	97.4	185.6

* Cultures grown in skimmilk for 24 hours at 42 C, stored at 10 C.

† Cysteic acid, threonine, glutamine, and proline were not detected in any culture.

reaching a pH of from 5.1–5.3. The free acidic α-amino compounds were measured after incubation of the cultures for 24 hours at 42 C, and after 2, 12, and 40 days of storage at 10 C.

The results of the studies of mixed strains of the three species are presented in table 3. It is apparent that the combination of strains gave a different pattern of amino acid accumulation than did the single strains of the test organisms recorded in table 1. This is especially apparent for the mixed strains of *S. thermophilus*, which gave a greater accumulation of free amino acids than did the single strain. In the mixed strain cultures, serine phosphate and glutamic acid were present at 2, 12, and 40 days of storage. The other acids were detected at certain times in some cultures, with none of the cultures containing measur-

TABLE 4. *The acidic free amino acid content of various mixed cultures of lactic acid starter organisms**

Age of Culture	Concentration of Acids in mg/1000 ml of Fermented Media							
	Serine phosphate	Taurine	Aspartic acid	Threonine	Serine	Asparagine	Glutamine	Glutamic acid
Lactobacillus lactis and *Streptococcus thermophilus* (mixed strains)								
2 days†								
12 days	60.3	0	0	0	0	0	0	87.65
40 days	3.3	0	0	0	0	17.4	0	14.2
Lactobacillus lactis and *Lactobacillus bulgaricus* (mixed strains)								
2 days†								
12 days	24.64	3.17	0	66.56	0	0	0	18.22
40 days	47.0	1.06	0	0	0	69.0	0	3.08
Lactobacillus bulgaricus and *Streptococcus thermophilus* (mixed strains)								
2 days†								
12 days	162.2	0	158.34	0	0	0	0	77.21
40 days	106.0	0	0	0	0	17.4	105.0	145.8
Lactobacillus lactis, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* (mixed strains)								
2 days	58.8	0	0	0	0	0	0	87.65
12 days	76.0	0	0	148.83	0	50.74	0	61.38
40 days	54.4	0	0	0	0	50.4	16.90	22.6

* Cultures grown in skimmilk for 24 hours at 42 C, stored at 10 C.

† No analysis.

able concentrations of cysteic acid, threonine, glutamine or proline.

The results of the various species grown in combination are shown in table 4. As was true for single strain cultures, there was a pronounced effect of one species on another. Threonine was not produced by any of the species grown alone, but the mixture of *L. lactis* and *L. bulgaricus* resulted in a rather high concentration of threonine at 12 days of storage. Glutamine was found only in the mixed culture of *L. bulgaricus* and *S. thermophilus* and in the mixture of these species with *L. lactis* but not with any species growing alone.

Control. A control consisting of only sterilized skimmilk media, incubated and stored at 10 C for the same length of time as in the other experiments, showed the presence of no acidic and amino compounds other than serine phosphate. This was detectable after incubation, then increased in concentration from 9.11 to 14.4 to 39.9 followed by a decrease to 18.2 to 14.2 and finally to 6.6 mg of serine phosphate per 1000 ml of skimmilk media at 12 hours, 3, 7, 18, 28, and 40 days respectively. No viable microorganism could be detected in one-ml samples of sterilized milk. At the present time, there is no apparent explanation for this interesting observation.

Discussion

The data presented in this paper suggest that the proteolytic activities of the starter organisms play an important part in bringing about the specific changes that take place in the ripening of Italian cheeses, Provolone and Romano. In this study, the proteolytic activities of these cultures were measured in terms of eleven free acidic α-amino compounds in skimmilk.

The proteolytic activity in cheese, in general, is due to the action of multiple enzyme systems from different sources: namely, the milk, rennet paste, and the microflora present. The results of this study suggest that each type or strain of lactic acid starter organism may produce different proteolytic end products when growing alone than when growing in combination with other strains of the same or different species. Single culture studies revealed that *L. lactis* V_{104} and *L. bulgaricus* V_4 were more active in their proteolytic activities than *S. thermophilus* T_5, while in mixed culture studies, the *S. thermophilus* showed more activity than both the *L. bulgaricus* and the *L. lactis*. The noticeable influence of one organism or group of organisms on one another, to bring about definite changes of the amino acid pattern as indicated in this investigation, may be explained on the basis of: (a) synergism between species or strains to bring about changes that neither of the species or strains could produce alone; (b) blocking of some reactions that lead to the liberation of certain amino acids; or (c) metabiosis, whereby the product of one organism may become a substrate for the other organism. Nurmikko (1952) has shown that symbiotic interrelationships do exist between different strains of lactic acid bacteria in a medium of known chemical composition.

Serine phosphate was produced in skimmilk media by all the lactic acid starter culture organisms under investigation, either growing alone or in combination with others. This acidic amino acid may have a biological significance in these cultures, but this is not clear at this time. It was also found in some sterile skimmilk samples initially and upon incubation at 42 C and storage at 10 C. The presence of serine phosphate may be due to chemical changes resulting from the sterilization of the skimmilk. The concentration of the serine phosphate in the sterile skimmilk control sample was almost one-fifth its concentration in any of the inoculated culture media. The presence of free amino acids has been reported by Block (1951) and by Van der Zant and Nelson (1954a) in a protein-free fraction of skimmilk. These investigators did not report the presence of serine phosphate acid in their studies. The role of serine phosphate is under investigation at the present time with the hope of securing more information about its liberation, utilization and significance.

Summary

The content of free acidic amino compounds in the protein-free fraction of skimmilk incubated at 42 C with Italian cheese starter organisms for 12 to 24 hours incubation and storage at 10 C for various lengths of time was investigated by ion exchange chromatography. The results showed an increase in amino acids in mixed cultures of various strains of *Lactobacillus lactis*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. Serine phosphate was present in all cultures due to bacterial action. It was also observed in some of the sterile skimmilk samples due to the effect of heat.

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